Diversity of Heterotrophic Protists from Extremely Hypersaline Habitats

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Heterotrophic protists (protozoa) are a diverse but understudied component of the biota of extremely hypersaline environments, with few data on molecular diversity within halophile ‘species’, and almost nothing known of their biogeographic distribution. We have garnered SSU rRNA gene sequences for several clades of halophilic protozoa from enrichments from waters of >12.5% salinity from Australia, North America, and Europe (6 geographic sites, 25 distinct samples). The small stramenopile Halocafeteria was found at all sites, but phylogenies did not show clear geographic clustering. The ciliate Trimyema was recorded from 6 non-European samples. Phylogenies confirmed a monophyletic halophilic Trimyema group that included possible south-eastern Australian, Western Australian and North American clusters. Several halophilic Heterolobosea were detected, demonstrating that Pleurostomum contains at least three relatively distinct clades, and increasing known continental ranges for Tulamoeba peronaphora and Euplaesiobystra hypersalinica. The unclassified flagellate Palustrimonas, found in one Australian sample, proves to be a novel deep-branching alveolate. These results are consistent with a global distribution of halophilic protozoa groups (~morphospecies), but the Trimyema case suggests that is worth testing whether larger forms exhibit biogeographic phylogenetic substructure. The molecular detection/characterization of halophilic protozoa is still far from complete at the clade level, let alone the ‘species level’.

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Introduction

Hypersaline habitats near or above 150 ‰ salinity are a type of extreme environment that is widely though sparsely distributed across the Earth, and that harbors a diverse biota adapted to high salinity waters (Javor 1989; Oren 2002). Extremely halophilic or halotolerant microorganisms capable of growth under these conditions are found across all domains of life (Kushner 1978). Halophilic bacteria are spread over 50 genera including Salinibacter and Halomonas, while halophilic archaea are distributed into 20 genera, mostly in the clade Haloarchaea (Oren 2002). The diversity of microbial eukaryotes is more poorly understood. The best known halophiles are photosynthetic algae, especially Dunaliella spp., and several forms of fungi (Javor 1989; Plemenitaš and Gunde-Cimerman 2005), but there is also a considerable

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if little-studied diversity of extremely halophilic or halotolerant protozoa (Cho 2005; Hauer and Rogerson 2005; Park et al. 2009; Ruinen 1938). For example, >25 apparently distinct morphospecies of protozoa have been observed even at ∼300‰ salinity, albeit mostly in microscopy surveys of natural samples or crude enrichments (representing ∼50 distinct sampling sites in total; see Supplementary Material table 1 in Park et al. 2009). Meanwhile, Galotti et al. (2014) reported 10 species of ciliate from a ∼150‰ salinity dilution of brine from a single salt pan (and many more species from lower salinity dilutions from several salt pans). Most free-living protozoa are phagotrophs that directly consume prokaryotes and other microbes (Laybourn-Parry 1992), so the potential or actual presence of protozoa in the most extreme categories of hypersaline habitats has important consequences for understanding the microbial ecology of these systems, especially if protozoa are abundant, as has been observed at least occasionally (Elloumi et al. 2009; Park et al. 2003).

Over several years, a number of obligately halophilic or highly halotolerant protozoa have been cultured from hypersaline environments and characterized using rRNA gene sequencing and (usually) electron microscopy examinations (Cho et al. 2008; Foissner et al. 2014; Kirby et al. 2015; Park et al. 2006, 2007, 2009; Park and Simpson 2011). They form at least 8 different genera, and belong to several distinct groups, including Stramenopiles (Halocafeteria), several groups of ciliates (Trimyema, Fabrea, and Platynematum), and several clades within Heterolobosea (Euplaeobiobyster, Pharyngomonas, and the Pleurostomum/Tulamoeba clade). The obligately halophilic protozoa cultured to date are genetically distinct from freshwater or marine protozoa, and often considered separate genera (Cho et al. 2008; Park et al. 2006, 2007, 2009; Park and Simpson 2011). Also, culture-independent sequencing-based studies of Mediterranean deep hypersaline anoxic basins (DHABs), the extremely hypersaline Lake Tyrrell in south-eastern Australia, and extremely hypersaline ponds in Spain and Chile have uncovered a number of 18S rRNA gene phylotypes representing protozoa that have not been detected in non-hypersaline samples (Alexander et al. 2009; Edgcomb et al. 2009; Heidelberg et al. 2013; Stock et al. 2012; Triadó-Margarit and Casamayor 2013). However, there is little overlap between the halophilic protozoa identified to date using culturing and the probable halophiles detected in sequence surveys, indicating that our knowledge of the diversity of halophilic protozoa remains poor. Furthermore, most of these recent studies of halophilic protozoa have used material from a single geographic region, or rarely two regions. As a result, there are few data on the sequence variation within the known groups of obligately halophilic protozoa, and almost nothing known about their global distribution, whether considering the level of groups (∼genera), of morphospecies, or of sequence-defined phylotypes. Since extremely hypersaline habitats are very small and sparse relative to marine, freshwater or soil systems, it is unclear whether generalisations about protist biogeography based on marine, freshwater or soil systems would necessarily apply.

In this study we investigated halophilic protozoa enriched from six sampling sites across western North America (California), south-eastern Australia (specifically Whyalla, in eastern South Australia), Western Australia, and central Europe (Poland), using 18S rRNA gene sequencing and light microscopy. At >125‰ salinity, all organisms identified on phenotype-based and molecular criteria, except one, were closely related to Halocafeteria, Trimyema, Pleurostomum, Tulamoeba or Euplaeobiobyster. Our data support the broad geographic spread of these halophilic groups (i.e. genera and/or morphospecies). A clear biogeographic signal was absent from internal phylogenies of the small flagellate Halocafeteria, but phylogenies of halophilic Trimyema, a ciliate which is several times larger, are consistent with biogeographic structuring, although our sampling is too limited to rigorously test this. We also obtained gene sequence data for the unclassified flagellate Palustrimonas, which proves to be a deep-branching alveolate and to represent a previously undocumented lineage of probable halophiles.

Results

Light Microscopy

Halocafeteria group

Cells were observed in cultures derived from 14 extremely hypersaline subsamples (260-322‰ or salt) from south-eastern Australia (Whyalla), Western Australia (Hutt Lagoon and Shark Bay), Europe (Wieliczka salt mine, Poland), and North America (Salton Sea periphery and Chula Vista solar saltern; Table 1). Halocafeteria cells were infrequently observed below 150‰ salinity (Table 1; unpublished observations). Cells were ovoid, 4-6 μm long and 2-4 μm wide, with two sub-equal flagella, each 1.5-2 times cell-length (Fig. 1 A-N). Cells usually either swam erratically with both
### Table 1. Summary of sampling locations and source salinity, and light microscopic observations and 18S rRNA gene sequences.

<table>
<thead>
<tr>
<th>Locations</th>
<th>Sampling date</th>
<th>Sample</th>
<th>Longitudes, Latitudes</th>
<th>Source salinity</th>
<th>Salinity of media</th>
<th>Light microscopic observations/18S rRNA gene sequences</th>
</tr>
</thead>
</table>
| Whyalla, SE Australia      | June, 2009    | Lake 4 | 33°2′4″S, 137°35′6″E       | 322 ppt         | 250 ppt           | ++/+/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/
Figure 1. Light micrographs of hypersaline biota from various salinity cultures (150‰ to 250‰ salinity media). All images are Differential Interference Contrast (DIC) micrographs. (A-G) *Halocafeteria* from Australia (A: Lake 6 at Whyalla, south-eastern Australia. B-D: H3, H4, and H5, Hutt Lagoon region, Western Australia. (E-G) S13B, S15B, and S16, Shark Bay, Western Australia). (H-K) *Halocafeteria* from North America (H-J: OH1A, OH2A, and OH3A from the Salton Sea region. K: SD1A from Chula Vista solar saltern). (L-N) *Halocafeteria* from Europe (respectively W1-24, WV10/2-300, and WVII 10/2-320, Wieliczka salt mine, Poland). (O) *Trimyema* from Lake 6 at Whyalla, south-eastern Australia, (P-R) *Trimyema* from S13B, S11B, and S15B in Shark Bay, Western Australia. S: *Trimyema* from SD1A in Chula Vista solar saltern, North America. (T) *Pleurostomum* from WVII 10/2-320 in Wieliczka salt mine, Poland. (U) *Pleurostomum* from OH2A, Salton Sea region, North America, (V-X) *Pleurostomum* from H4, H5, and H6, Hutt Lagoon, Western Australia. (Y) *Palustrimonas* from S15B in Shark Bay, Western Australia. Scale bar at bottom right represents 10 µm for all images. See Table 1 for source salinities.
flagella beating, or attached to substrates by the posterior flagellum, while the anterior flagellum created a feeding current.

**Trimonyma** group
Cells were observed in six extremely hypersaline subsamples with source salinities 180-322‰ from south-eastern Australia (Whyalla), Western Australia (Shark Bay) and North America (Salton Sea and Chula Vista solar saltarm; Table 1). This *Trimonyma* group was not found below 150‰ salinity (Table 1; unpublished observations). The cell body was 20-35 μm long and 10-15 μm wide, with an elongately ellipsoidal profile (anterior end bluntly pointed; posterior end rounded), a prominent caudal cilium, a cytostome near the anterior end, and a semicircular arrangement of oral cilia (Fig. 1O-S).

**Heterolobosea**
We observed *Pleurostomum*-like flagellates in material sourced from five subsamples of >280‰ salinity from Western Australia (Hutt Lagoon), Europe (Wieliczka salt mine), and North America (Salton Sea; Table 1). Cells were spindle-shaped and 12-20 μm long, with parallel homodendritic flagella that inserted subapically. A lateral rostrum appeared near the flagellar insertion (Fig. 1T-X) and a cytostomal structure extended in a longitudinal spiral from below the rostrum to near the posterior end. These cells correspond closely to reports of *Pleurostomum flabellatum* by Patterson and Simpson (1996) and Park et al. (2007).

**Palustrimonas**
Cells corresponding closely to *Palustrimonas yokkeensis* (Patterson and Simpson 1996; Ruinen 1938) were observed in material from one subsample from Shark Bay, Western Australia (S15B; source salinity 280‰). Cells were 9-12 μm long, with a slender ovate shape (Fig. 1Y). The two unequal flagella inserted subapically. The anterior flagellum was approximately cell-length and inserted into a small subapical groove. The posterior flagellum was approximately 2.5 times cell-length and inserted into a larger longitudinal groove. No vacuoles containing prokaryote prey were observed. Cells rotated counter-clockwise during swimming. No gliding motility was observed.

**Phylogenetic Analyses**

**Stramenopiles: Halocafeteria**
Thirty-one 18S rRNA gene sequences from Stramenopiles were obtained from the 14 subsamples in which *Halocafeteria* cells were documented by microscopy, plus three additional samples (Fig. 2B, Table 1). These spanned all the major locations examined (all three continents; both south-eastern Australia and Western Australia). All of the near-full-length stramenopile sequences showed very high similarity to *Halocafeteria seosinensis* EHF34 (Park et al. 2006; 97.5%-100% identity). Most differences amongst sequences were in variable regions, especially the V4 region. As a consequence, sequences were up to 5.6% dissimilar to one another across the interior sequence region of ~630 bp that was obtained for all sequences, since this included the V4 region.

Phylogenetic analyses included our new sequences from Australia, North America and Europe samples, plus new sequences from Korea associated with the type strain EHF34, and one additional *Halocafeteria*-like sequence from California, North America (GenBank sequence GO31629). No *Halocafeteria*-like sequences were detected in published environmental sequence surveys from hypersaline sites (e.g. Alexander et al. 2009; Edgcomb et al. 2009; Heidelberg et al. 2013; Triadé-Margarit and Casamayor 2013), in contrast to the cases of *Trimonyma* and Heterolobosea (see below). In maximum likelihood (ML) and Bayesian phylogenetic analyses the *Halocafeteria* group formed a clade with high ML bootstrap support (ML: 89%) or posterior probability (PP) 1 (Fig. 2A). This clade was the sister group to the halotolerant (and probably moderately halophilic sensu Oren 2008) strains ME280100 and ME13100 (Park and Simpson 2010), with high support (ML: 83%, PP: 1; Fig. 2A).

We examined phylogenetic structure in the *Halocafeteria* group with outgroups excluded (Fig. 2B). The relationships amongst *Halocafeteria* clones were incompletely resolved, however the most strongly supported clades (sensu Wilkinson et al. 2007) did not represent groupings defined by region or continent (Fig. 2B). For example, sequence WVII 10/2 300 clone 21/1 (Europe; Poland) grouped with sequences from the Korean *H. seosinensis* strain EHF 34 (ML: 100%, PP: 1), while other sequences from Europe (WVII 10/2 320 clones 12/2 and 12/3) grouped with Western Australia and western North America sequences (ML: 72%, PP: 0.72), and still other clones from Europe (also from WVII 10/2 320) were most closely related to a different western North America clone, Salton Sea OH2A clone 8/2 (ML: 80%, PP: 1). Meanwhile, a different Salton Sea sequence, OH1A clone 7/2, formed a strong clade with two sequences from Western Australia, S13 clone 5/33 and H3 clone 4/3 (ML: 93%, PP: 1;
Figure 2. Maximum likelihood phylogenetic trees of 18S rRNA gene sequences from the hypersaline Halo cafeteria group. (A) Outgroup-rooted phylogeny of Halo cafeteria based on sequences >650bp (27 ingroup sequences). Out-groups are the closely related halotolerant stramenopiles ME280100 and ME13100, plus other representative bicosoecids (MESS21, Cafeteria, and Caecitellus). (B) Phylogeny estimated for Halo cafeteria sequences only (34 sequences total) inferred from the middle part of the 18S rRNA gene including the variable V4 region (positions 583-1209 in Halo cafeteria seosinensis EHF34). Bootstrap values (>55%) from the maximum likelihood analysis are shown at the nodes. Solid circles indicate a Bayesian posterior probability of 1 (posterior probabilities <0.9 not shown). Superscript numbers represent different clones from the same subsample. Open circles: subsamples from Australia, Filled squares: subsamples from Europe, Open squares: subsamples from North America, † and ††; additional 18S rRNA gene sequences of monoeukaryotic cultures, Halo cafeteria seosinensis and WVII 10/2 320, respectively, amplified by a high-fidelity PrimeSTAR® HS DNA polymerase (Takara, Tokyo, Japan).

see Fig. 2B). A single moderately supported clade (ML: 69%, PP: 1) grouped some clones from Western Australia (Hutt lagoon H4 clones 3/3, 3/5 and 3/9 and H6 clone 11/13; Shark Bay S15B clone 16/10) with a clone from Whyalla, south-eastern Australia (Lake 4 clone 1). However, the dataset as a whole had a high representation of Australian sequences, and there was a substantial probability that any given clade of this size would contain only Australian sequences by chance alone (0.067 when near-identical clones from the same culture are collapsed).

Trimyema group
We obtained 18S rRNA gene sequences from each of the six isolates in which Trimyema was observed by microscopy (seven sequences reported). These were from Whyalla and Shark Bay in south-eastern Australia and Western Australia respectively, and from the Salton Sea region and the Chula Vista solar saltern in North America (Table 1). These sequences were 95.0%-99.8% identical to Trimyema species, including the halophile T. koreanum, and environmental sequences LT85_L8 and cLA14E04.
Figure 3. Maximum likelihood phylogenetic tree of 18S rRNA gene sequences showing the evolutionary position of the hypersaline Trimyema group. The in-group Trimyema groups include 21 sequences from cultures, environmental samples, and the seven sequences reported in this study. Out-groups are Lechriopyla, Plagiopyla, and environmental sequence (AB252767) in the class Plagiopylae. Note that three unclassified environmental sequences are from deep-sea hypersaline basins in the Mediterranean Sea. Bootstrap values (>55%) from maximum likelihood analysis are shown at the nodes. Solid circles indicate a Bayesian posterior probability of 1 (posterior probabilities <0.9 not shown). Open circles: subsamples from Australia, Open squares: subsamples from North America.

Phylogenies of 18S rRNA gene sequences included a clade within Trimyema that contained all strains from hypersaline environments, with maximum ML bootstrap support and Bayesian PP (Fig. 3). Most hypersaline Trimyema sequences fell into three major lineages (Groups I, II and III) with moderate-to-high ML bootstrap support (74%-100%) and PP (0.91-1.0; Fig. 3). Group I
included the Korean type strain of T. koreanum and all Trimyema sequences from Mediterranean DHABs. Group II contained all new sequences from the two North American sites. Group III included sequences from south-eastern Australia only, both our sequence from Whyalla, South Australia and recently determined environmental sequences from Lake Tyrrell, Victoria (Heidelberg et al. 2013). The remaining three 18S rRNA gene sequences, from three different Western Australian (Shark Bay) subsamples, were extremely similar (99.7%-99.8% identity) and formed a weakly supported Group IV (ML: 47% and PP: 0.77). A consistent topology was inferred when outgroups were excluded (not shown). Three other environmental sequences (BB2D04, cLA13C09, and TB1B11) from Mediterranean DHABs formed a strong clade (ML: 96%, PP: 1; Fig. 3) that robustly fell outside of the Trimyema clade, confirming that they represent an independent clade of putative halophiles (Trimyema monophyly received 93% ML bootstrap support and PP 1).

Heterolobosea

The five heterolobosean sequences obtained were most similar to one or other group of known halophiles. Sequences similar to Pleurostomum flabellatum (88.9%-98.9% identity) were retrieved from cultures from the Wieliczka salt mine (Europe; Poland), and Hutt Lagoon (Western Australia), in which Pleurostomum cells had been observed by microscopy. A sequence was also obtained from another subsample from Hutt Lagoon (H1) where they had not been observed. No Pleurostomum-like sequences were retrieved from Hutt Lagoon H5 and H6, or Salton Sea OH2A material, despite microscopy records. Single sequences very similar to the type strains of Tulamoeba peronaphora (99.1% identity), and Euplaesiobystra hypersalinica (99.3% identity) were obtained from Hutt Lagoon sample H3 (Western Australia), without accompanying microscopy observations.

The five 18S rRNA gene sequences (WII 10/2 320 clone 6, H1 clone 14, H4 clone 3/48, H3 clone 4/7, and H3 clone 4/8) were placed within Heterolobosea with very high support (ML: 99%, PP: 1; Fig. 4). All sequences except H3 clone 4/8 fell within a maximally supported Pleurostomum + Tulamoeba clade of halophiles/halotolerant forms. WII 10/2 320, H1 clone 14, and H4 clone 3/48 were specifically included within a clade containing P. flabellatum, with maximum ML bootstrap support or PP 1 (Fig. 4). WII 10/2 (from Poland; Europe) was very closely related to the 98.9% identical P. flabellatum strain EHF1 from Korea. H1 clone 14, and H4 clone 3/48 from Hutt Lagoon, Western Australia were more distantly related, though the latter was closely related to environmental sequence LT19_L3 from Lake Tyrrell (south-eastern Australia). H3 clone 4/7 (Hutt Lagoon, Western Australia) formed a tight clade with the 99.1% identical type strain of Tulamoeba peronaphora, from Korea.

The final heterolobosean sequence, H3 clone 4/8 (Hutt Lagoon, Western Australia), was placed in a very tight Euplaesiobystra clade with maximum ML bootstrap support and PP (Fig. 4). The other Euplaesiobystra isolates were from Korea and Europe (Portugal), and all share 99.3% identity.

Palustrimonas

An 18S rRNA gene sequence of the previously unclassified Palustrimonas was obtained from the culture in which it was observed by microscopy (from the S15B subsample from Shark Bay in Western Australia). In 18S rRNA gene phylogenies Palustrimonas branched within a robust alveolates clade (ML: 97%, PP: 1; Fig. 5). Interestingly, Palustrimonas did not branch within either of the two main clades within alveolates, Ciliophora (ciliates) or Myzozoa (including dinoflagellates, Apicomplexa, Perkinsids, Colpodellids, Chromera and Vitrella). Instead, Palustrimonas joined the recently examined taxa Acauonomas and Colponema as a deep branching lineage affiliated with Myzozoa, although the clade containing all of these taxa lacked statistical support (ML: 54, PP: 0.70). The relative branching order of Palustrimonas, Acauonomas, Colponema and Myzozoa sensu stricto was also poorly resolved.

Discussion

Diversity of Halophilic Protozoa

By combination of cultivation, microscopy and molecular phylogenetic techniques, elements of the protozoan biota of hypersaline habitats were identified. All species studied here are usually reported from extreme hypersaline environments (i.e. 150‰ to saturated brine) where marine or freshwater organisms have little presence (Javor 1989). The range of source salinities for our observations of Halocafeteria, Trimyema, Pleurostomum, Tulamoeba, and Euplaesiobystra are largely within the salinity ranges for their growth determined previously for monokaryotic strains cultivated in the laboratory (Cho et al. 2008; Park et al. 2006, 2007,
Figure 4. Maximum likelihood phylogenetic tree of 18S rRNA gene sequences showing the evolutionary position of hypersaline *Pleurostomum*, *Tulamoeba*, and *Euplaesiobystra* groups in Heterolobosea. The isolates reported in this study are represented in bold. Out-groups are Euglenozoa (11 sequences), Jakobida (four sequences), and *Tsukubamonas globosa*. Bootstrap values (≥55%) from maximum likelihood analysis are shown at the nodes. Solid circles indicate a Bayesian posterior probability of 1 (posterior probabilities <0.9 not shown).

This is consistent with them being active in the extremely hypersaline habitats from which they were enriched.

The majority of distinct forms (at a 95% sequence identity threshold) encountered were heteroloboseans. This echoes the observation that more than a third of the morphospecies observed at ~300‰ salinity or higher in previous microscopy-based studies were probably heteroloboseans (Park et al. 2009). It is notable, however, that heterolobosean sequences are rare or absent in 18S rRNA/DNA clone libraries from extremely hypersaline lakes and DHABs (Alexander et al. 2009; Edgcomb et al. 2009; Heidelberg et al. 2013; Stock
Figure 5. Maximum likelihood phylogenetic tree of 18S RNA gene sequences showing the phylogenetic position of Palustrimonas relative to 81 other eukaryotes, including representatives of the major clades of alveolates. Bootstrap values (>50%) from maximum likelihood analysis are shown at the nodes. Solid circles indicate a Bayesian posterior probability of 1 (posterior probabilities <0.9 not shown).

et al. 2012; Triadó-Margarit and Casamayor 2013). Heidelberg et al. (2013), for example, reported a single heterolobosean sequence, ‘LT19_L3’ (GenBank sequence KC486154), from hypersaline Lake Tyrrell in Victoria, south-eastern Australia. This sequence proves to be most closely related to Pleurostomum, and it shares 97.7% identity with a Pleurostomum sequence from Hutt Lagoon in Western Australia. This very limited detection of Heterolobosea may reflect a true rarity in natural habitats, with the high relative frequency of culturing them being due to culture bias. However, it probably also reflects the divergent nature of heterolobosean 18S rRNA gene sequences, and (in DNA-based surveys) the common presence of group I introns in halophilic Heterolobosea (Harding et al. 2013; Park et al. 2007; Park and Simpson 2011). Environmental sequencing strategies that are designed...
with Heterolobosea in mind and/or more direct observations of their abundance in natural samples would be timely.

We detected three different lineages belonging to the Pleurostomum clade. One culture, WVL 10/2 320 clone 6, from Wieliczka salt mine, Poland, could be considered an isolate of the species *P. flabellatum*, as originally cultured from a Korean solar saltern (Park et al. 2007). The other two *Pleurostomum* cultures are probably best considered different species, based on their substantial sequence dissimilarity. Interestingly, the *Pleurostomum*-like environmental sequence LT19_L3 (see above) was derived from a 127‰ salinity sample (Heidelberg et al. 2013). Park et al. (2007) reported that *P. flabellatum* strain EHF1 cultures failed to grow below 200‰ salinity. Assuming that LT19_L3 came from an active form (rather than a cyst or moribund cell), this is consistent with the 18S rRNA gene evidence that it represents a different species. There are also six nominal species of *Pleurostomum* described by microscopy alone (*P. caudatum*, *P. salinum*, *P. gracile*, *P. parvulum*, *P. flabellatum*, and *P. turgidum*), all of which have been observed in high salinity brines (Namyslowski 1913; Patterson and Simpson 1996; Ruinen 1938). In short, various evidence indicates that the *Pleurostomum*-Tulamoeba clade contains a substantial diversity at even a conservatively-defined species level, and represents an adaptive radiation of halophilic/halotolerant organisms (see also Kirby et al. 2015).

This study includes the first sequence data for the unclassified protist *Palustrimonas*, which was cultivated from 280‰ salinity material from Shark Bay, Western Australia. Interestingly, the previous observations of *Palustrimonas yorkeensis* (basionym *Phyllomitus yorkeensis*) were also from Australian material with salinities between 160‰ and saturation (Patterson and Simpson 1996; Ruinen 1938). Growth at these extreme salinities has not been proven for *Palustrimonas*, but we maintained our strain in mixed culture for several months at 250‰ salinity (Park, unpublished), strongly suggesting that it is a true halophile. *Palustrimonas* is probably a predator of eukaryotes (Patterson and Simpson 1996), and is morphologically similar to colpodellids, which are obligate eukaryotic predators that have been observed often in hypersaline environments (Heidelberg et al. 2013; Patterson and Simpson 1996; Post et al. 1983; Simpson and Patterson 1996). However, colpodellids have a distinctive life cycle that includes a specialized and obligate reproductive cyst (see Simpson and Patterson 1996) that has not been observed for *Palustrimonas*. In fact, our phylogenetic trees show that *Palustrimonas* is not closely related to colpodellids, including colpodellid sequences from the hypersaline Lake Tyrrell (Heidelberg et al. 2013; LT35_P9 and LT85_A4 in Fig. 5). Therefore *Palustrimonas* represents a novel lineage of (probably) halophilic protozoa.

*Palustrimonas* falls into a very deep-branching position within Alveolata, similar to that occupied by colponemids (not to be confused with colpodellids) and Acavomonas, both of which were only recently characterized using molecular sequencing (Tikhonenkov et al. 2014). *Palustrimonas* bears some resemblance to colponemids, which are biflagellate cells with a ventral groove associated with the posterior flagellum, and are often predators of other eukaryotes (Tikhonenkov et al. 2014). *Palustrimonas*, colponemids, and Acavomonas are key for understanding the evolutionary history of Alveolata, which is an important and famously diverse clade (Tikhonenkov et al. 2014). Taxon sampling and data for these organisms are very sparse at present, and further examinations of *Palustrimonas* would be of broad value.

**Patterns of Occurrence**

The most commonly detected taxon, *Halocafeteria*, was present in most samples with salinities of 132‰ or higher. *Halocafeteria* was first cultured from 300‰ salinity water from a Korean solar saltern (Park et al. 2006). This strain, EHF34, grew optimally at 150‰ salinity and 35°C, but would grow across a wide range of salinities (i.e. >75-363‰, Park et al. 2006). This flexibility may help explain the common occurrence of *Halocafeteria* in hypersaline samples. Nonetheless, no sequences from *Halocafeteria* were found within any of the clone libraries from Mediterranean anoxic deep-sea basins (DHBs) with salinities of 280‰ to 340‰ (Alexander et al. 2009; Edgcomb et al. 2009; Stock et al. 2012), nor from a benthic salt crust sample from the Lake Tyrrell basin, Australia (Heidelberg et al. 2013). This might be due to very low density of *Halocafeteria* in some/most natural environments at some/most times (which does not conflict with our observations, which are based on enrichments) and/or an inability to grow in anoxic conditions. Another possible explanation is that *Halocafeteria* may be unable to grow in very high concentrations of certain ions. The waters in Lake Tyrrell contained very high sulfate concentrations (15 g l⁻¹, Macumber 1992). Many organisms have difficulties growing in the high MgCl₂ concentrations of DHAB brines (Hallsworth et al. 2007, Park 2012).
reported that *Halocafeteria* strain EHF34 did not grow in media with a high Mg:Ca ratio (100) and high sulfate concentrations (≥11 g l⁻¹) under otherwise ideal conditions (i.e. 150‰ salinity and 35 °C, with no prey limitation).

By contrast, *Trimyema* is the only clade of halophilic protozoa that has been detected both in surface hypersaline waters (of 172‰+ salinity; including Lake Tyrrell) and in DHABs (Alexander et al. 2009; Cho et al. 2008; Edgcomb et al. 2009; Heidelberg et al. 2013). This probably reflects the fact that *Trimyema* spp. are anaerobes (Wagener and Pfennig 1987). The occurrence of this halophile group both in surface habitats and DHABs is further evidence that DHABs do indeed support an active eukaryote biota that is distinct from the biota of the overlying marine system (Alexander et al. 2009). Further, it indicates that this biota has historical links to surface hypersaline habitats, rather than representing independent (and presumably recent) local adaptation by marine taxa.

Extremely hypersaline habitats are sparsely distributed across the globe, raising the question of whether the larger microbiota that inhabit them (e.g. protozoa) have dispersed rapidly amongst them or whether they exhibit biogeography at some meaningful level of group resolution, such as morphospecies, or narrower sequence-defined groups. At the broad level of light microscopy identities, this study supports the global distribution of culturable morphospecies of halophilic protozoa, with even the rarely-encountered groups of Heterolobosea included here now generally recorded from multiple continents (see also Kirby et al. 2015; Park and Simpson 2011). However, we also documented substantial 18S rRNA gene sequence divergence within the *Halocafeteria* and halophilic *Trimyema* morphotypes, such that internal phylogenies for each could be inferred and compared to geographic origins.

We did not find consistent evidence of segregation by geographic region for subclades of the small flagellate *Halocafeteria*. If there is biogeographic structure within this group, it must be manifest at a finer scale than could be evaluated with the sampling depth in our study, and even with better sampling, might only be detectable using a more rapidly evolving molecular marker, e.g. ITS sequences (see Bass et al. 2007). By contrast 18S rRNA gene phylogenies recover subclades of hypersaline *Trimyema* that may well have geographically restricted distributions. Hypersaline *Trimyema* Group II was only found in North America (i.e. Salton Sea and Chula Vista solar salterns: ~150 km apart), while hypersaline *Trimyema* Group III was only detected in south-eastern Australia (Whyalla and Lake Tyrrell; ~ 550 km apart). The three 18S rRNA gene sequences from *Trimyema* found in Shark Bay, Western Australia are very similar to each other, and might represent another geographically restricted cluster (Shark Bay is >2,400 km from Whyalla, the closest south-eastern Australia site). Clearly, this inference is provisional given the small number of sites and isolates examined, but we believe it represents a hypothesis worth testing. Firstly, it would be valuable to test whether the clusters identified here are robust when more discriminating phylogenetic markers are considered, for example ITS sequences (Bass et al. 2007). Secondly, further sampling could confirm or refute the proposition that these clades are indeed restricted to particular regions, and establish whether there is geographic substructure within Group I, which currently includes a Korean isolate amongst sequences from Mediterranean DHABs. This would also help to test the possibility that the apparent biogeographic signal is actually due to habitat differences that partly co-vary with the current geographic sampling.

There is some evidence that species (or other reasonably distinct clades) of free-living ciliates might be restricted to particular geographic locations (Foissner 2006). Many cases of apparent endemism of ciliate morphospecies have been falsified by improved global sampling (e.g. Esteban et al. 2001). Nonetheless, the freshwater ciliate *Tetrahymena thermophila*, which is similar in size to halophilic *Trimyema*, has been detected only in eastern North America, despite the extensive global sampling of *Tetrahymena* (Foissner 2006; Kher et al. 2011; Nanney et al. 1998). On the other hand, other similarly distinct clades of *Tetrahymena* are known to exist on more than one continent (see Table 1 in Kher et al. 2011), and it must be noted that the 18S rDNA divergence between subgroups of halophilic *Trimyema* is greater than between some nomenclatural species of *Tetrahymena*. Interestingly, Finlay et al. (2006) did not recover distinct American and European clusters within a subclade of the *Cyclidium glaucoma* morphospecies that was isolated from hypersaline samples of 40-93‰ salinity (that is, moderately hypersaline, rather than extremely hypersaline, as in the current study).

**Summary, and Evolutionary Implications**

This study broadly supports the global distribution of readily-enriched halophilic protozoa groups, that is, morphospecies or tight 18S rDNA clades. Most of these groups of halophilic protozoa have now
been observed from multiple continents, with the most commonly encountered organisms having the broadest recorded ranges. This is consistent with dispersal dominating over local adaptation in the ultimate evolutionary origins of obligately halophilic protozoa. This is despite the relatively large number of independent origins of obligate halophily already documented among the protozoa (e.g. Park and Simpson 2011), which on its own suggests that adopting obligate halophily may be a relatively 'easy' evolutionary transition for organisms that are already marine (noting that this inference is different to, and does not conflict with, the inference that freshwater-to-marine transitions among microbes are relatively uncommon and thus evolutionarily 'challenging'—see Logares et al. 2009).

Further, the demonstration that Palastrimonas represents another ‘novel’ clade of probable halophiles is another indication that we still have a substantially incomplete inventory of major groups of halophilic protozoa. This is also suggested by a high number of light microscopy records of hypersaline-dwelling protozoa that are unlikely to belong to sequence-characterised clades (see Park et al. 2009), as well as the several 18S rDNA clades that are only known as environmental sequences from hypersaline sites, especially DHABS (Stock et al. 2012). Until this catalog is more complete we will be poorly positioned to understand general adaptations to halophily amongst protozoa, and the level of distinctiveness of the halophile communities in different kinds of extreme hypersaline habitats.

**Methods**

**Crude cultures**: Samples were taken from natural and artificial sites in North America (Southern California) in June 2009, Australia (Western Australia; south-eastern Australia) in June 2009, and Europe (Poland) in June 2008 (Table 1). In all, 25 distinct water bodies with salinities of 125‰ to saturation were sampled, with each sampled once only (Table 1). Samples were collected without concentration in previously sterilized collection containers from the shore (i.e. from shallow water at the margin of the water body). Where possible, samples of 5 ml total volume containing 1-2 ml volume of surface sediment was also collected from the sediment-water interface, using a sterilized metal scoop, or sterile disposable pipette. Samples were directly inoculated (incubation volume: 100-150 μl) into 5 ml of 100-250‰ salinity media made by dilution of AS medium (300‰ artificial salinity; 272 g NaCl, 7.6 g KCl, 17.8 g MgCl₂, 1.8 g MgSO₄, 7H₂O, 1.3 g CaCl₂ 1⁻¹ water; Park 2012) with sterile water, supplemented by a sterile barley grain to support prokaryote growth. Crude cultures were incubated at 22-25 °C for at least 2 weeks. Live cells were observed with differential interference microscopy using a Zeiss Axiobrath 200 M microscope equipped with an Axiocam MR digital camera, before extraction of nucleic acids from cultures.

**18S rRNA gene sequencing**: Nucleic acids from the isolates were prepared using a DNeasy Blood and Tissue Kit (Qiagen, Maryland, USA). Amplification of 18S rRNA genes was performed using standard polymerase chain reaction (PCR) protocols, with different combinations of the widely used general eukaryotic-specific primers EuKα (5’-AACCTGGTGTGATCGCTGAGT-3’), 42F (5’-CTCAARGAYTAAGCCATGCA-3’), EuKB (5’-TGATCTTCTGT- GCAGGTCACCTAG-3’), 1496R (5’-CACCTCGGACTA- GCCTGTGTA-3’), and 18S’ R (5’-GGAATGACTTACCTCTGACA-3’), where the first two are forward primers and the latter three are reverse primers, and/or specific primers for the 18S rRNA genes from particular previously cultured halophiles (Table 2). The reaction mixtures contained 50-100 ng of DNA, 0.2 mM deoxynucleoside triphosphate, 0.5 μM each primer, 2.0 mM MgCl₂, and 2.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, USA). The 18S rRNA genes of Halocafeteria culture SVII 10/2 320, and the previously isolated Halocafeteria seosiensis strain EHF34 (Park et al. 2006), were also amplified using high-fidelity PrimeSTAR® HS DNA polymerase (Takara, Tokyo, Japan). The annealing temperatures were 55 °C (pairs of general eukaryotic primers) or 60 °C (combinations of one general eukaryotic primer and one cultured halophile specific primer, Table 2), and reactions were cycled 40 times. The size of each PCR product (~1.4 to 2.0 kb) was determined by gel electrophoresis. Amplicons were normally cloned into a pGEM-T Easy vector, at least 10 positive clones per sample were partially sequenced, and different positive clones were completely sequenced using various sequencing primers. The broad identities of the 18S rDNA gene sequences were determined by BLASTN searches against the Genbank nr database. In the cases where multiple similar sequences were determined (Halocafeteria and Trimyema), the great majority of differences were within variable regions (indicative of true sequence differences rather than in-vitro/in-silico errors). Putative chimeras were identified by using the Bellephoron program (Huber et al. 2004). Three potentially chimeric sequences were identified within the Halocafeteria dataset, with subsequent manual inspection revealing another four sequences where chimerism could not be excluded. To be conservative, these sequences were reported as partial sequences only (the ~630 bp V4-containing region; for which there is no evidence of chimerism) and excluded from the full-length phylogenetic analysis (see below). The reported 18S rDNA gene sequences have the Genbank accession numbers KT210042-KT210046.

**Phylogenetic analyses**: We constructed four groups of datasets for phylogenetic analysis. 1) 18S rRNA genes sequences similar to the stramenopile Halocafeteria were included with sequences from the type strain of Halocafeteria seosiensis (EHF34) and two similar environmental sequences (27 sequences total), plus 8 sequences closely related to Halocafeteria as an outgroup (retaining 1,468 unambiguously aligned sites). A dataset was also constructed that excluded outgroup sequences, and included only the 627 unambiguously aligned sites present in all partial sequences (positions 583-1209 in the type strain of H. seosiensis), which encompassed the information-rich V4 variable region (34 sequences total). 2) Sequences similar to Trimmymea (Ciliophora, Plagiopylea) were included with Trimmymea sequences from freshwater, marine, and hypersaline environments, along with very similar environmental sequences (21 sequences total), plus three plagiopyle outgroups and three additional unclassified environmental sequences from Mediterranean DHABS (EU446401, JF308263, and FJ000077), with 1,642 unambiguously aligned sites retained. A dataset that included only halophilic Trimmymea
Table 2. New clade-specific 18S rRNA primers used in this study (F - Forward; R -Reverse).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S-HS451F</td>
<td>GATACGGAACCCA(A/T)(A/C)GGGTTT</td>
<td>Specific to Halocafeteria and ME280100 + ME13100 halotolerant clade</td>
</tr>
<tr>
<td>18S-Tri1446R</td>
<td>CA(A/G)TTCCCCACTAGA(T/C)AC(G/A)G</td>
<td>Specific to Trimyema</td>
</tr>
<tr>
<td>18S-Ple1520R</td>
<td>CCAACTATCGCTGATCCTAAAT</td>
<td>Specific to Pleurostomum flabellatum</td>
</tr>
<tr>
<td>18S-Ple1825R</td>
<td>CTCGCATCTTCTTCTGGTTC</td>
<td>Specific to Pleurostomum flabellatum</td>
</tr>
</tbody>
</table>

and related environmental sequences was also assembled (1,666 sites retained). 3) Sequences similar to Pleurostomum, Tulamoeba and Euplaesiobystra were included with diverse other Heterolobosea (49 sequences total), with 16 sequences from Jakobida, Euglenozoa and Tsukubamonas as outgroups (1,041 well-aligned sites retained). 4) A dataset containing 47 sequences from alveolates, plus 35 from other ‘short-branching’ eukaryote clades was assembled to evaluate the phylogenetic position of Palustrimonas (1,438 unambiguously aligned sites retained). All datasets were aligned and masked by eye. These alignments are available on request.

Phylogenetic trees for each dataset were inferred by maximum-likelihood (ML) analysis and by Bayesian analysis. The GTR + gamma + I model of sequence evolution was selected for all datasets using MrModeltest 2.2 (Nylander 2004), and used for all analyses. The ML tree was estimated using RAxML-Vi-HPC v7 (Stamatakis 2006) with the GTRGAMMMA model setting, 200 random starting taxon addition sequences, and statistical support estimated using bootstrapping with 10,000 replicates. The Bayesian analysis was conducted in MrBAYES 3.2 (Huelsenbeck and Ronquist 2001) with two independent runs of four chains each running for 5,000,000 generations (burn-in 550,000 generations, average standard deviation of split frequencies threshold = 0.03), with heating parameter 0.2 and sampling frequency 0.01.

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